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Brief Communication

MEASUREMENT OF URINARY 8-EPI-PROSTAGLANDIN $F_{2\alpha}$, A NOVEL INDEX OF LIPID PEROXIDATION IN VIVO, BY IMMUNOAFFINITY EXTRACTION/GAS CHROMATOGRAPHY-MASS SPECTROMETRY. BASAL LEVELS IN SMOKERS AND NONSMOKERS

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Abstract—8-Epi-prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$) is an F_2-isoprostane recently identified as a marker of free radical-catalyzed lipid peroxidation in vivo and potential mediator of oxidative damage. Currently, endogenous 8-epi-$PGF_{2\alpha}$ is measured by gas chromatography-mass spectrometry after lengthy sample preparation. We extracted and purified 8-epi-$PGF_{2\alpha}$ in one step from biological samples on immunoaffinity columns prepared with an anti-8-epi-$PGF_{2\alpha}$ antiserum, raised in our laboratory. Quantitation was done by stable-isotope dilution gas chromatography/negative-ion chemical ionization mass spectrometry, with selected ion recording. Carboxylate anions of the pentafluorobenzyl ester trimethylsilyl ether derivative of 8-epi-$PGF_{2\alpha}$ and [$^2H_4$]8-epi-$PGF_{2\alpha}$ were monitored (m/z 569 and 573). Basal urinary excretion of 8-epi-$PGF_{2\alpha}$ can be accurately and rapidly measured by this method. Under normal conditions rats (n = 30) excreted 2.18 ± 0.68 ng/24 h. In healthy nonsmoking young volunteers, urinary excretion of 8-epi-$PGF_{2\alpha}$, measured three times on alternate days, was fairly constant (CV 2–10%). Nonsmokers excreted significantly less 8-epi-$PGF_{2\alpha}$ than age-matched smokers (8.08 ± 2.3 vs. 18.40 ± 4.77 ng/h/1.73 m^2; n = 6; $p < 0.005$), as reported by others using different methods.

Keywords—Isoprostanes, 8-Epi-prostaglandin $F_{2\alpha}$, Lipid peroxidation, Cigarette smoking, Immunoaffinity chromatography, Gas chromatography-mass spectrometry, Urine, Free radicals

INTRODUCTION

Isoprostanes are recently discovered prostaglandin-like products formed in vivo by free-radical catalyzed nonenzymatic peroxidation of arachidonic acid.[1,2] These compounds are formed in situ esterified in phospholipids, then released in free form.[3] Circulating and urinary levels of F_2-isoprostanes are high in patients with pathologies involving oxidant stress or in animal models of oxidative damage.[1,4] F_2-isoprostanes are formed in plasma and low density lipoprotein (LDL) exposed to oxidative stress[5,6] in vitro, suggesting that these compounds may also be markers of LDL oxidation in vivo.

8-Epi-$PGF_{2\alpha}$, one of the most abundant isoprostanes formed in vivo,[1,7] is a potent renal and pulmonary vasoconstrictor.[2,8,9] Formation of 8-epi-$PGF_{2\alpha}$ was found to be increased in animal models of renal ischemia-reperfusion and pulmonary oxygen toxicity, and it has, therefore, been proposed as a mediator of oxidative damage in these organs.[8,10]

As compared to the other methods,[11] selective measurement of F_2-isoprostanes appears to be more reliable for evaluating lipid peroxidation in vivo.[12,13] The validity of this approach has now been further confirmed by data showing that smokers, who are exposed to the oxidants of cigarette smoke, have increased levels of urinary 8-epi-$PGF_{2\alpha}$,[4] urinary F_2-isoprostane metabolites,[14] as well as free and esterified plasma F_2-isoprostanes.[14] All these parameters were increased to much the same extent—about 200%—in smokers compared to age-matched nonsmokers.

Selective measurement of individual isoprostanes in biological samples is difficult because they must be

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separated not only from their numerous isomers[15] but also from endogenous prostaglandins and their metabolites.[16] The methods so far used for purifying 8-epi-$PGF_{2\alpha}$ before GC-MS measurement are based on several chromatographic steps, including time-consuming TLC.[4,17] We describe here a faster method based on immunoaffinity extraction/gas chromatography-mass spectrometry for selective measurement of urinary 8-epi-$PGF_{2\alpha}$. The method proved suitable for accurate measurement of basal urinary excretion of 8-epi-$PGF_{2\alpha}$ in humans and rats. We then measured 8-epi-$PGF_{2\alpha}$ in age- and sex-matched smokers and nonsmokers.

EXPERIMENTAL PROCEDURES

Materials

8-Epi-$PGF_{2\alpha}$ and 3,3′,4,4′-[$^2H_4$]-8-epi-$PGF_{2\alpha}$ were purchased from Cayman Chemicals (Ann Arbor, MI). Pentafluorobenzyl bromide, *N,N*-diisopropylethylamine and bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) were obtained from Fluka (Buchs, Switzerland). Methoxylamine hydrochloride (MOX) was from Pierce (Rockford, IL). Incomplete Freund's adjuvant, *N*-acetylmuramyl-L-alanyl-D-isoglutamine, keyhole limpet hemocyanin, *N*-hydroxybenzotriazole, *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide hydrochloride were from Sigma Aldrich (Milano, Italy). CNBr-activated Sepharose 4B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

Anti-8-epi-$PGF_{2\alpha}$ antisera

Antisera against 8-epi-$PGF_{2\alpha}$ were raised in three male HY/Cr rabbits (Charles River, Calco, Italy), as described previously for 11-dehydro-TXB_2.[18] Immune-stimulating complexes (ISC) containing 8-epi-$PGF_{2\alpha}$, and *N*-acetylmuramyl-L-alanyl-D-isoglutamine [muramyl dipeptide (MDP)], coupled to keyhole limpet hemocyanin (KLH) were prepared by the following steps: (1) dissolve 1 mg 8-epi-$PGF_{2\alpha}$ in 50 μl dimethylformamide (DMF); (2) add 1.55 μmol MDP in 50 μl DMF; (3) add 4.7 μmol *N*-hydroxybenzotriazole in 94 μl DMF and 4.7 μmol *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide hydrochloride in 94 μl DMF, incubating for 1 h at room temperature; (4) add the resulting solution in 10 30-μl aliquots to a dark glass vial containing KLH (2.0 mg in 1.2 ml of 0.1 M $NaHCO_3$ buffer, pH 8.5) under vigorous stirring, incubating for 3 h at room temperature, to form amide bonds between carboxy groups in 8-epi-$PGF_{2\alpha}$ and MDP and amino groups in KLH; (5) add 1.5 ml PBS; (6) store the resulting ISC solution as 100-μl aliquots at −20°C.

Each rabbit was immunized with 100 μl ISC diluted to 1 ml with PBS and emulsified with 1 ml incomplete Freund's adjuvant. The immunization was repeated at weeks 3 and 6 and every 4 weeks thereafter. Antibody titer was monitored by ELISA (using an 8-epi-$PGF_{2\alpha}$-BSA conjugate as coating antigen) in test bleeds taken from the ear artery 8 d after each booster injection. Titer was defined as the maximal antiserum dilution giving a positive signal (sample-to-negative ratio > 2) in the assay against 8-epi-$PGF_{2\alpha}$-BSA (100 ng/well) after subtracting the nonspecific response of pretreatment serum. Titers of the antisera collected after the second boost until the time of writing (6th boost) were in the range $1:10^5$–$1:10^6$.

The specificity of the different antisera was tested 'on column': the IgG fraction was purified from each antiserum and immobilized as described below, and the resulting immunosorbents were tested by GC-MS for their ability to selectively extract 8-epi-$PGF_{2\alpha}$ from urine or from phosphate buffer (0.05 M, pH 7.4) spiked with 8-epi-$PGF_{2\alpha}$, $PGF_{1\alpha}$, 6-keto-$PGF_{1\alpha}$, PGE_2, and TXB_2 (1 ng each).

Immunosorbents

The IgG fraction was isolated from the three antisera by Protein A Fast Flow (Pharmacia, Uppsala, Sweden) and coupled to CNBr-activated Sepharose-4B as recommended by the manufacturer, using 5 mg protein/ml swollen gel. Typically, 6–8 mg IgG were obtained per ml of serum. The resulting immunosorbents (A,B,C) were suspended in 0.05 M phosphate buffer containing 0.02% merthiolate and stored at 4°C in the dark until used. Standard columns for urine extraction were prepared with 100 μl settled immunosorbent, while 1.2-ml columns were used to extract 30–50 ml urine samples for full-scan GC-MS analysis of immunoextracts (see below).

Immunoaffinity extraction

The extraction procedure, described in detail elsewhere,[19] was slightly modified as follows. Urine samples corresponding to about 4-min diuresis for human urine (1–8 ml) and about 5-h diuresis for rat urine (2–5 ml) were diluted to 20 ml with phosphate buffer (0.05 M, pH 7.4) containing [$^2H_4$] 8-epi-$PGF_{2\alpha}$ and filtered. Samples were percolated through the immunoaffinity column containing the anti-8-epi-$PGF_{2\alpha}$ immunosorbent. The column was washed with 20 ml distilled water and eluted with 3 × 0.5 ml acetone:water (95:5, v/v), as described.[19] The columns were repeatedly used after appropriate washings (20 ml distilled water, 10 ml acetone:water (95:5, v/v), 20 ml distilled water). The eluate was dried under a stream of air and

derivatized to form the pentafluorobenzyl ester (PFB), trimethylsilyl ether (TMS) derivative of 8-epi-PGF$_{2\alpha}$. For samples where other prostanoids were to be measured and for urine extracts to be analyzed by full-scan GC-MS, another reaction step was added to form the methoxime derivative (MO). The reaction was carried out as follows: (1) add 30 μl pentafluorobenzyl bromide:acetonitrile (1:20, v/v) and 5 μl diisopropylethylamine to the dried sample (5 min at 40°C); (2) dry under a stream of air; (3) repeat steps 1 and 2; (4) add 50 μl BSTFA (15 min at 60°C). For the MO derivative add 20 μl MOX reagent plus 30 μl acetonitrile (60°C, 1 h) after step 3, then dry.

Gas chromatography/negative-ion chemical ionization mass spectrometry (GC-NICIMS)

A Finnigan 4000 quadrupole mass spectrometer, equipped with a Vector-Two data system (Teknivent Corp, St. Louis, MO), and directly interfaced with a DANI (Monza, Italy) 6500 gas chromatograph was used. GC operating conditions were: Easy 52 (5% phenyl–95% methyl-polysiloxane) fused-silica capillary column (25 m length; 0.32 mm i.d.; 0.12 μm film thickness; Analytical Technology, Cernusco sul Naviglio, Italy); oven temperature, isothermal at 160°C for 1 min, then programmed from 160°C to 300°C at 15°/min; septumless injection mode using a Programmed Temperature Vaporizer (PTV, DANI, Monza, Italy) at 50°C for 6 s (solvent split) then at 300°C (splitless); helium as carrier gas. NICI operating conditions were: selected ion recording (SIR) of carboxylate anions (M-181, loss of CH_2C_6H5), m/z 569 for 8-epi-PGF$_{2\alpha}$ and m/z 573 for [$^2H_4$] 8-epi-PGF$_{2\alpha}$; ammonia as reagent gas; electron energy, 100 eV.

Standard curves

A standard curve was obtained for each sample set with increasing amounts (0–4 ng) of 8-epi-PGF$_{2\alpha}$ in the presence of 1 ng [$^2H_4$] 8-epi-PGF$_{2\alpha}$, by plotting the [$^2H_0$]/[$^2H_4$] 8-epi-PGF$_{2\alpha}$ peak area ratio against the amount of unlabeled 8-epi-PGF$_{2\alpha}$ (typically, r = 0.9998).

Urine collection

Six-hour urine was collected between 10.00 and 16.00 h from volunteers working at our Institute. Six

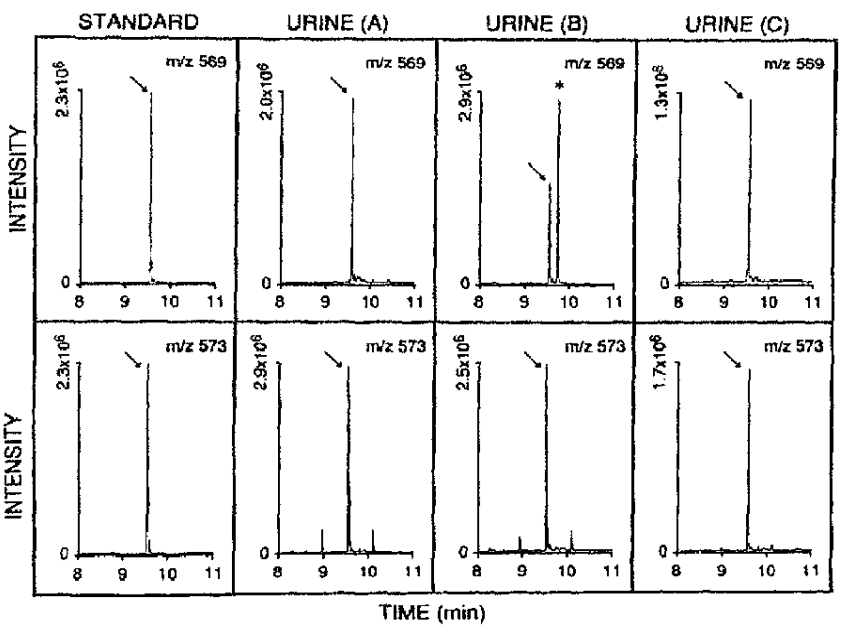

Fig. 1. Selected ion recording chromatograms of authentic 8-epi-PGF$_{2\alpha}$ and urinary 8-epi-PGF$_{2\alpha}$ extracted by different immunosorbents (A,B,C). Identical urine samples (4 ml of a urine pool obtained from nonsmoking subjects under normal conditions) were immunoextracted after addition of 1 ng [$^2H_4$] 8-epi-PGF$_{2\alpha}$. Arrows indicate 8-epi-PGF$_{2\alpha}$ (m/z 569, retention time 9.59 s) or [$^2H_4$] 8-epi-PGF$_{2\alpha}$ (m/z 573, retention time 9.60), and the asterisk (*) indicates PGF$_{2\alpha}$.

male nonsmokers and six male smokers (age: 29.8 ± 3.7 and 29.8 ± 5.8 years, respectively; body surface area: 1.98 ± 0.17 m^2 and 2.01 ± 0.07 m^2, respectively) were recruited. Smokers reportedly consumed 19 ± 7 cigarettes/d. Three urine collections were made on alternate days for each subject. Volunteers were apparently healthy and had not taken drugs or vitamin supplements in the preceding 10 d.

Urine was also collected for 24 h from thirty male Sprague–Dawley CD COBS rats (Charles River, Calco, Italy; 200 g body weight) kept in metabolic cages with free access to food and water. [Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directives 86/609, OJ L 358, 1, Dec. 12, 1987; NIH Guide for the Care and Use for Laboratory Animals, NIH Publication No. 85-23, 1985).]

Urine samples were frozen and stored at −20°C until analyzed.

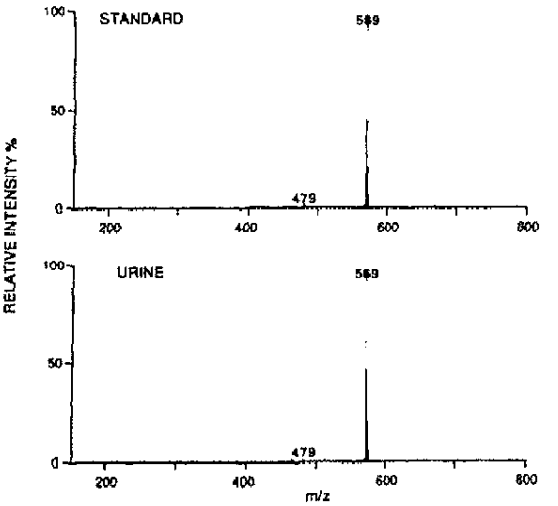

Fig. 2. Negative-ion chemical ionization mass spectra of authentic 8-epi-$PGF_{2\alpha}$ and 8-epi-$PGF_{2\alpha}$ immunoextracted from human urine and derivatized to PFB ester TMS ether.

RESULTS

Immunosorbent evaluation by GC-NICIMS

Figure 1 presents the selected ion recording chromatograms of urine extracts (4 ml pooled urine, spiked with 1 ng [$^2H_4$] 8-epi-$PGF_{2\alpha}$) obtained from anti-8-epi-$PGF_{2\alpha}$-immunosorbents A, B and C, showing that all immunosorbents extracted endogenous 8-epi-$PGF_{2\alpha}$ and only immunosorbent B extracted $PGF_{2\alpha}$ in addition.

Extraction of a mixture of authentic 8-epi-$PGF_{2\alpha}$ and other prostanoids (1 ng each, with deuterated analogues added after extraction to quantify recovery) indicated that none of the immunosorbents extracted 6-keto-$PGF_{1\alpha}$, PGE_2 or TXB_2 (recovery < 1%), but they all efficiently extracted 8-epi-$PGF_{2\alpha}$ (recovery 90–100%). Immunosorbent B also extracted $PGF_{2\alpha}$ (recovery 80%). Immunosorbent C was used for routine urine analysis.

GC-NICIMS analysis of urinary immunoextracts (without addition of internal standard) from all three sorbents made it possible to record full scan mass spectra of putative endogenous 8-epi-$PGF_{2\alpha}$. These spectra were identical to that of authentic 8-epi-$PGF_{2\alpha}$ (Fig. 2). In immunoextracts of both human and rat urine obtained from all three immunosorbents, treated or not with MOX reagent, we noted an unidentified endogenous crossreactant eluting 8 s after 8-epi-$PGF_{2\alpha}$, with complete peak separation. Its NICI mass spectrum showed a prominent ion cluster at m/z 639 and no ions at m/z 569 or 573 (< 0.2% of m/z 639). In our conditions this crossreactant did not interfere with quantitation of 8-epi-$PGF_{2\alpha}$.

Immunoaffinity extraction of 8-epi-$PGF_{2\alpha}$

Deuterium labeled and unlabeled 8-epi-$PGF_{2\alpha}$ were identically recovered, as demonstrated by extracting 0.2 , 0.5, 2.0, or 10.0 ng [$^2H_0$] 8-epi-$PGF_{2\alpha}$ in the presence of 2 ng [$^2H_4$] 8-epi-$PGF_{2\alpha}$ from buffer. The [$^2H_0$]/[$^2H_4$] peak area ratios of extracted (y) or unextracted (x) samples were the same ($y = 1.005x - 0.015$, $r = 0.999$).

Accuracy of the assay was evaluated after extraction and GC-MS analysis of triplicate 4-ml aliquots of a human urine pool (five healthy males) spiked with increasing amounts (0, 0.05, 0.25, and 1.00 ng/ml) of 8-epi-$PGF_{2\alpha}$ and a constant amount (1.0 ng) of [$^2H_4$] 8-epi-$PGF_{2\alpha}$. The amount (ng/ml) recovered (y) corresponded to the amount added (x) plus endogenous level ($y = 1.005x + 0.180$, $r = 0.999$). Precision was calculated by extracting six 4-ml aliquots of the same urine pool without adding exogenous 8-epi-$PGF_{2\alpha}$. The mean concentration ±SD was 0.187 ± 0.009 ng/ml (CV 4.8%).

The validity of the assay was also assessed using increasing urine volumes with a constant amount of internal standard. Different sized samples (0.5, 1.0, 4.0, and 8.0 ml) of a urine pool were carried through the analysis after addition of 1 ng [$^2H_4$] 8-epi-$PGF_{2\alpha}$. The amount of 8-epi-$PGF_{2\alpha}$ recovered (ng, y) in-

creased linearly with sample volume (ml, x) ($y = 0.190x - 0.018$, $r = 0.999$).

Human urine

The method described above was used to evaluate basal urinary excretion of 8-epi-PGF$_{2\alpha}$ in 12 healthy male volunteers (six nonsmokers and six smokers) (Fig. 3). Smokers excreted significantly more 8-epi-PGF$_{2\alpha}$ than nonsmokers [mean ± SD of individual mean values (three collections per subject): 18.40 ± 4.77 vs. 8.08 ± 2.3 ng/h/1.73 m^2, $p < 0.005$, Student's *t*-test]. Repeated urine collections ($n = 3$) for each subject on different days showed that urinary excretion of 8-epi-PGF$_{2\alpha}$ was fairly constant in nonsmokers (Fig. 3): mean intrasubject variation (CV) was 5.2 ± 3.9% (range 2.1–10.5%) in nonsmokers but 11.8 ± 8.0% (range 4.5–24.3%) in smokers.

Rat urine

Basal excretion values of 8-epi-PGF$_{2\alpha}$ in rats ($n = 30$) was 2.18 ± 0.68 ng/24 h or 0.205 ± 0.080 ng/mg creatinine.

DISCUSSION

Measurement of F$_2$-isoprostanes in general, and of 8-epi-PGF$_{2\alpha}$ in particular, is a new way of assessing lipid peroxidation and oxidant stress in vivo.[1,13] The limitations and biological significance of this approach have been reviewed recently.[13] Although all the data appear to confirm its validity, the model is relatively recent and needs characterizing better. Unfortunately, few laboratories have been involved so far in measuring isoprostanes, probably because of the complex methodology.[16] We have, therefore, developed a simpler method for measuring endogenous 8-epi-PGF$_{2\alpha}$.

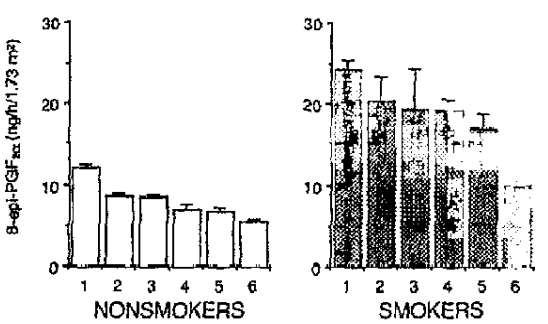

Fig. 3. Urinary excretion of 8-epi-PGF$_{2\alpha}$ in nonsmoking and smoking healthy volunteers. Each bar represents the mean individual value (± SD) based on 6-h urine collections on different days ($n = 3$).

Immunoaffinity extraction/GC-MS is a powerful tool for rapid purification and specific measurement of trace compounds in complex biological matrices.[19] With the method described here to raise anti-8-epi-PGF$_{2\alpha}$-antisera, we obtained enough antibodies to prepare efficient immunoaffinity columns about 3 months from the first immunization. GC-MS of immunoextracted material enabled us to test directly for the selectivity of the immunosorbents against other prostanoids in pure form as well as against the myriad of endogenous compounds present in urine. Our antibodies are specific enough to provide interference-free selected ion recording chromatograms without preextracting or purifying the samples, allowing accurate measurement of urinary 8-epi-PGF$_{2\alpha}$.

It is convenient to measure 8-epi-PGF$_{2\alpha}$ (or F$_2$-isoprostane metabolites) in urine for a number of reasons: besides the fact that sampling is noninvasive, sample handling and storage are simpler than for plasma or other lipid-containing tissues or fluids, where artifactual formation of isoprostanes must be avoided.[13] We are now testing the procedure to extend its use to free and esterified 8-epi-PGF$_{2\alpha}$ measurement in plasma, tissues, and other biological fluids.

The basal urinary excretion of 8-epi-PGF$_{2\alpha}$ measured by our method in normal volunteers was consistent with that reported by others using different extraction/purification methods before GC-MS.[6] Our data also confirm the recently reported twofold increase in 8-epi-PGF$_{2\alpha}$ excretion in smokers.[6,14] Interestingly, Morrow et al.[14] found that urinary excretion of enzymatic metabolites of F$_2$-isoprostanes, strongly correlated to the level of free circulating F$_2$-isoprostanes in smoking and nonsmoking volunteers, was 2.1 times higher in smokers. Urinary excretion of 8-epi-PGF$_{2\alpha}$ was very similarly increased in smokers (2.1 vs. 2.3 times, as measured by Catella et al.[4] and ourselves, respectively). This suggests that, at least in smokers, levels of free or esterified circulating F$_2$-isoprostanes are equally reflected by urinary F$_2$-isoprostane metabolites or 8-epi-PGF$_{2\alpha}$.

Individual 8-epi-PGF$_{2\alpha}$ excretion tended to be quite constant in nonsmokers, with a relatively low interindividual variation. This finding suggests that individual 'normal' levels, in the absence of manifest oxidant injury, may result from physiological biochemical event(s) occurring at a constant rate with by-production of free radical-derived reactive species, possibly because of scant local scavenging capacity of the antioxidant defense system. Moore et al.[6] recently suggested that basal 8-epi-PGF$_{2\alpha}$ production in vivo may result from lipid peroxidation triggered by a

chain of chemical events starting with the reaction of endothelium-derived nitric oxide and superoxide to form peroxynitrite.

In conclusion, this method represents a significant advance in terms of rapidity and simplicity over other GC-MS methods for selectively measuring 8-epi-$PGF_{2\alpha}$. The technique will make it easier to monitor the effects of antioxidants, drugs, or dietary manipulations on in vivo formation of 8-epi-$PGF_{2\alpha}$. In view of its specificity, this analytical procedure may also help better define the validity and limitations of 8-epi-$PGF_{2\alpha}$ as a marker of oxidant stress in vivo in experimental and clinical settings.

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ABBREVIATIONS

BSA—bovine serum albumin
BSTFA—bis-(trimethylsilyl)-trifluoroacetamide
CV—coefficient of variation
DMF—dimethylformamide
ELISA—enzyme-linked immunosorbent assay
GC-MS—gas chromatography-mass spectrometry
IgG—immunoglobulin G
ISC—immune-stimulating complexes
KLH—keyhole limpet hemocyanin
LDL—low density lipoprotein
MDP—*N*-acetylmuramyl-L-alanyl-D-isoglutamine [muramyl dipeptide]
MOX—methoxylamine hydrochloride
NICI—negative-ion chemical ionization
PBS—phosphate-buffered saline
PFB—pentafluorobenzyl
8-epi-$PGF_{2\alpha}$—8-epi-prostaglandin $F_{2\alpha}$
SIR—selected ion recording